TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2015) 39: 298-309

doi:10.3906/bot-1401-66

Seed micromorphology in *Dactylorhiza* Necker ex Nevski (Orchidaceae) and allied genera

Roberto GAMARRA[1,*], Pablo GALÁN[2], Henrik Ærenlund PEDERSEN[3], Emma ORTÚÑEZ[1], Ernesto SANZ[1]

[1]Department of Biology, Faculty of Sciences, Autonomous University of Madrid, Madrid, Spain
[2]Department of Vegetal Production: Botany and Vegetal Conservation, E.U.I.T. Forestal, Polytechnic University of Madrid, Madrid, Spain
[3]Botanical Garden, Natural History Museum of Denmark, University of Copenhagen, Copenhagen K, Denmark

Received: 23.01.2014 • **Accepted:** 06.08.2014 • **Published Online:** 16.03.2015 • **Printed:** 10.04.2015

Abstract: Seeds of 21 taxa of the genera *Dactylorhiza* (incl. *Coeloglossum*), *Gymnadenia* (incl. *Nigritella*) and *Pseudorchis* were examined by light microscope and SEM. Qualitative and quantitative characters were analyzed. In *Dactylorhiza*, the seeds are fusiform, but some populations of *D. viridis* show clavate seeds. According to the ornamentation of the periclinal walls, 3 types of seeds are recognized in this genus. The considerable variation in the seed coat ornamentation pattern in the taxa belonging to *majalis*, *maculata*, and *praetermissa* groups of the genus *Dactylorhiza* is congruent with the genetic processes that occurred during the history of this genus. *Gymnadenia* shows clavate seeds with stout and straight to slightly curved anticlinal walls, although these are straight to wavy in some taxa considered previously as *Nigritella*. These taxa also show low values of seed length. *Pseudorchis* has fusiform seeds without ornamentation in the periclinal walls and fine anticlinal walls. Our study supports the inclusion of the former genus *Coeloglossum* in *Dactylorhiza*.

Key words: *Dactylorhiza*, *Coeloglossum*, *Gymnadenia*, *Nigritella*, *Pseudorchis*, SEM, testa, periclinal walls

1. Introduction

Within the tribe Orchideae (Orchidaceae), one of the most controversial groups belongs to the so-called "digitate tuber" clade (Pridgeon et al., 1997), which includes the genera *Dactylorhiza* Necker ex Nevski, *Gymnadenia* R.Br., and *Pseudorchis* Ség. In the molecular phylogenetic analyses published by Pridgeon et al. (1997) and corroborated by Bateman et al. (2003), *Coeloglossum* Hartm. and *Nigritella* Rich. were nested in *Dactylorhiza* and *Gymnadenia*, respectively. Accordingly, *Coeloglossum* and *Nigritella* were synonymized in *Genera Orchidacearum* (Pridgeon et al., 2001). However, based on either morphological or molecular data, other authors (Aedo and Herrero, 2005; Delforge, 2006; Devos et al., 2006) have maintained them as independent genera.

Dactylorhiza is distributed from Europe and the Mediterranean across temperate Asia to the Himalayas, Japan, and Alaska (Pridgeon et al., 2001; Sheviak et al., 2002). Generally, *Dactylorhiza* is considered a complex genus, and there is still no consensus about the delimitation of species and their classification into aggregates or groups (Averyanov, 1990; Delforge, 2006; Pillon et al., 2007). In his extensive review of the genus, Averyanov (1990) recognized 75 species within 4 sections, subdivided into subsections and aggregates. Pridgeon et al. (2001) considered about 50 species in the total range of the genus, including the only species of *Coeloglossum* in it. Delforge (2006) reported 60 species for Europe, North Africa, and the Middle East, within 7 groups (*iberica*, *sambucina*, *incarnata*, *majalis*, *traunsteineri*, *praetermissa*, *maculata*). However, according to the species concept advocated by Pedersen (1998), far fewer species should be recognized (Pillon et al., 2006; Pedersen 2010). Even the overall structure in some of the currently prevailing classifications is in conflict with the patterns that have emerged from recent molecular studies. For example, the members of an allotetraploid complex in Greece that Hedrén et al. (2007), based on allozymes, AFLPs, and plastid DNA data, found to be extremely closely related (partly even synonymous) were assigned to 3 different species groups in Delforge (2006).

Nowadays, the inclusion of *Coeloglossum viride* (L.) Hartm. in the genus *Dactylorhiza* is generally accepted. This species has a holarctic-boreal distribution, extending from North America to Japan (Delforge, 2006). Morphologically, it is distinguished from the rest of the genus *Dactylorhiza* by the spur, which is short and nectariferous, and the petals and sepals, which are joined

* Correspondence: : roberto.gamarra@uam.es

in a connivent helmet, while the labellum is bifid and the rostellum bears 2 bursicles rather than 1 (Szlachetko and Rutkowski, 2000; Aedo and Herrero, 2005).

Gymnadenia comprises about 30 species distributed in Eurasia and along the western coast of North America (Pridgeon et al., 2001). While members of *Gymnadenia* s.s. have the labellum oriented downwards, taxa with the labellum oriented upwards have frequently been treated as a distinct genus, *Nigritella* (Teppner and Klein, 1998; Hedrén et al., 2000).

Pseudorchis has a circumboreal distribution, from Canada to the Kamchatka Peninsula (Pridgeon et al., 2001). Only *P. albida* (L.) A. & D.Löve is recognized, but its 2 subspecies (Reinhammar, 1998) are sometimes treated at species level. Based on molecular phylogenetic analyses, Pridgeon et al. (1997, 2001) and Bateman et al. (2003) recognized *Pseudorchis* as an independent genus, more closely related to *Platanthera* Rich. than *Gymnadenia*, where it was assigned by Delforge (2006).

From a cytogenetic point of view, all the genera mentioned above have the same chromosome base number (x = 20), and different mechanisms of aneuploidy and speciation processes by hybridization and polyploidy have been reported (Cauwet-Marc and Balayer, 1984; Amich et al., 2007). Apart from the yet unknown *Coeloglossum* × *Nigritella* and *Coeloglossum* × *Pseudorchis*, all possible hybrid combinations between these putative genera have been recorded in the field (Aedo and Herrero, 2005; Delforge, 2006; Bateman, 2009). A particularly remarkable case is the so-called *Gymnigritella runei* Teppner & E. Klein - tetraploid *Gymadenia conopsea* × *Nigritella nigra* hybrids that form self-reproducing populations in northern Sweden (Hedrén et al., 2000). Based on data from diverse molecular markers, patterns of allopolyploidization have also been well documented within *Nigritella* (Hedrén et al., 2000) and *Dactylorhiza* (Hedrén, 1996; Pedersen, 2006; Hedrén et al., 2007; Pillon et al., 2007).

Previous studies on orchid seeds have demonstrated the taxonomic value of quantitative and qualitative characters (Clifford and Smith, 1969; Barthlott, 1976; Arditti et al., 1979; Chase and Pippen, 1988). In addition, recent publications (Tsutsumi et al., 2007; Gamarra et al., 2007, 2008, 2010, 2012) have emphasized the strong correlation between seed micromorphology and molecular analyses, as reconstructed using DNA sequence data.

Tohda (1983) analyzed 13 species of the genus *Dactylorhiza* (sub syn. *Dactylorchis*), recognizing 3 groups based on quantitative characters of the seed, and suggested that this differentiation was indicative of 3 major phylogenetic lineages within the genus. He also established another division in 3 different groups based on the surface sculpture of the testa. Averyanov (1990) distinguished 4 types of seeds (*Dactylorhiza incarnata*-type, *D. maculata*-type, *D. romana*-type, and *D. aristata*-type) according to the ornamentation pattern of the periclinal walls.

In the genus *Nigritella*, Haas (1977) mentioned that the testa cells are reticulated in *N. nigra* (possibly *N. nigra* subsp. *austriaca* Teppner & E.Klein), but smooth in *N. miniata*. Teppner and Klein (1994) described the seeds of *N. gabasiana* and *N. nigra* subsp. *iberica* without ornamentation in the periclinal walls and with enlarged medial to basal cells in the second taxa. Later, Sáez (2004) found the same characteristics in both taxa.

Using light microscopy, Mrkvicka (1994) analyzed quantitative and qualitative data of the seeds of *Coeloglossum viride*, 7 taxa of *Dactylorhiza*, 4 of *Gymnadenia*, and *Pseudorchis albida*.

Our study continues the research into seed micromorphology in the tribe Orchideae (Gamarra et al., 2007, 2008, 2010, 2012). The present study aimed to survey the seed coat micromorphology of the genus *Dactylorhiza* and allied genera using SEM and light microscopy, and provide new insights into its potential taxonomic value. One of the primary objectives was to describe their seed morphology and identify seed characters based on qualitative and/or quantitative data.

2. Materials and methods

We analyzed seeds of 21 taxa belonging to the genera *Dactylorhiza*, *Gymnadenia*, and *Pseudorchis*.

Seeds were obtained from mature capsules collected in the field or from herbarium specimens (C, M, MA, MAF, MAUAM, US, W, acronyms according to Thiers, 2012). A list of voucher specimens and localities is given in the Appendix (on the journal's website), with scientific names according to Govaerts et al. (2014). Fresh seeds were dried for at least 1 month and stored in small paper envelopes.

The samples were mounted on SEM stubs and coated with gold in a sputter-coater (SEM Coating System, Bio-Rad SC 502). Then 10 to 15 seeds of each sample were examined with a Philips XL30, with a filament voltage of 20 kV. Qualitative data such as general morphology, apical to basal cells, and characteristics of the anticlinal and periclinal walls were analyzed, and selected images were recorded.

An average of 30 seeds from each specimen were analyzed with a light microscope, previously mounted with polyvinyl alcohol. Seed size (length and width), the ratio length/width, and the number of cells along the longitudinal axis were recorded. All the taxa were measured except *D. cantabrica*, because we did not find a high number of seeds in good condition. Seed color was described in subjective terms. Seed and embryo dimensions were analyzed statistically using analysis of variance (ANOVA) and Tukey's test performed with SPSS 21.0 for Windows (IBM Corp. Released 2012, New York).

The terminology and methods were adopted from Arditti et al. (1979), Barthlott and Ziegler (1981), and Chase and Pippen (1988).

3. Results

Qualitative characters were analyzed by SEM in all the populations of the taxa included in the Appendix (on the journal's website). Generally, the seeds show short apical and basal cells, and the medial cells are rectangular and more elongated. For each taxon analyzed, the qualitative characters are consistent except for some group of species mentioned below. As shown in Table 1, the main differences between taxa were found in the following characters: seed shape, ornamentation of the periclinal walls, and shape of the anticlinal walls.

In *Dactylorhiza*, all the seeds were fusiform (Figure 1A) except in some populations of *D. viridis*, as mentioned below. Anticlinal walls with raised lamella were found in all the taxa (Figure 1B). There were infrageneric differences in the ornamentation of the periclinal walls.

No ornamentation was found in the periclinal walls of *Dactylorhiza incarnata* or *D. viridis* (Figure 1B). In the latter, we found 3 types of seed shape, fusiform (Figure 1C) in the northern populations of America, fusiform to clavate in northern Russia (Figures 1D and 1E), and clavate (Figure 1F) in the southern populations of Europe.

The taxa of the *sambucina* group (*D. sambucina*, *D. romana* subsp. *romana*, *D. romana* subsp. *guimaraesii*, *D. cantabrica*, *D. insularis*) show straight to slightly curved anticlinal walls in the apical cells (Figure 2A), but they are generally straight in the medial and basal cells (Figure 2B). The main feature of this group is the reticulate sculpture on the periclinal walls, with anastomosed trabeculae (Figure 2C). However, *D. insularis* shows parallel trabeculae, perpendicular to the longitudinal axis, but rarely anastomosed trabeculae (Figure 2D). The same pattern was found in a reduced number of seeds in *D. cantabrica*.

A third group is characterized by the presence of different kinds of trabeculae in the periclinal walls of the same seed: anastomosed (Figure 3A and 3B), transversal

Table 1. Qualitative data of the analyzed species.

Species	Seed shape	Ornamentation of the periclinal walls	Anticlinal walls (apical pole)	Anticlinal walls (medial cells)
Dactylorhiza incarnata	Fusiform	Smooth	Straight	Straight
D. viridis	Fusiform to clavate	Smooth	Straight	Straight
D. sambucina	Fusiform	Reticulated	Straight to slightly curved	Straight
D. cantabrica	Fusiform	Transversal trabeculae	Straight to irregularly curved	Straight to irregularly curved
D. insularis	Fusiform	Transversal trabeculae	Straight to slightly curved	Straight
D. romana subsp. *romana*	Fusiform	Reticulated	Straight to slightly curved	Straight to slightly curved
D. romana subsp. *guimaraesii*	Fusiform	Reticulated	Straight to slightly curved	Straight
D. majalis subsp. *majalis*	Fusiform	Different types of trabeculae	Straight	Straight
D. alpestris	Fusiform	Different types of trabeculae	Curved to sinuous	Curved to sinuous
D. elata	Fusiform	Different types of trabeculae	Straight	Straight
D. cordigera	Fusiform	Different types of trabeculae	Straight	Straight
D. purpurella	Fusiform	Different types of trabeculae	Straight to slightly curved	Straight to slightly curved
D. maculata subsp. *maculata*	Fusiform	Different types of trabeculae	Straight	Straight
D. maculata subsp. *caramulensis*	Fusiform	Different types of trabeculae	Straight	Straight
D. foliosa	Fusiform	Different types of trabeculae	Straight	Straight
D. fuchsii	Fusiform	Transversal trabeculae	Straight	Straight
Gymnadenia conopsea	Clavate	Smooth	Straight	Straight
G. densiflora	Clavate	Smooth	Straight to slightly curved	Straight
G. odoratissima	Clavate	Smooth	Straight to slightly curved	Straight
G. austriaca	Clavate	Smooth	Straight to sinuous	Straight to slightly curved
G. austriaca var. *gallica*	Clavate	Smooth	Curved to sinuous	Straight to slightly curved
G. corneliana	Clavate	Smooth	Straight to slightly curved	Straight to slightly curved
G. miniata	Clavate	Smooth	Straight to slightly curved	Straight to slightly curved
G. nigra	Clavate	Smooth	Straight to curved	Straight to curved
Pseudorchis albida	Fusiform	Smooth	Straight to slightly curved	Straight to slightly curved

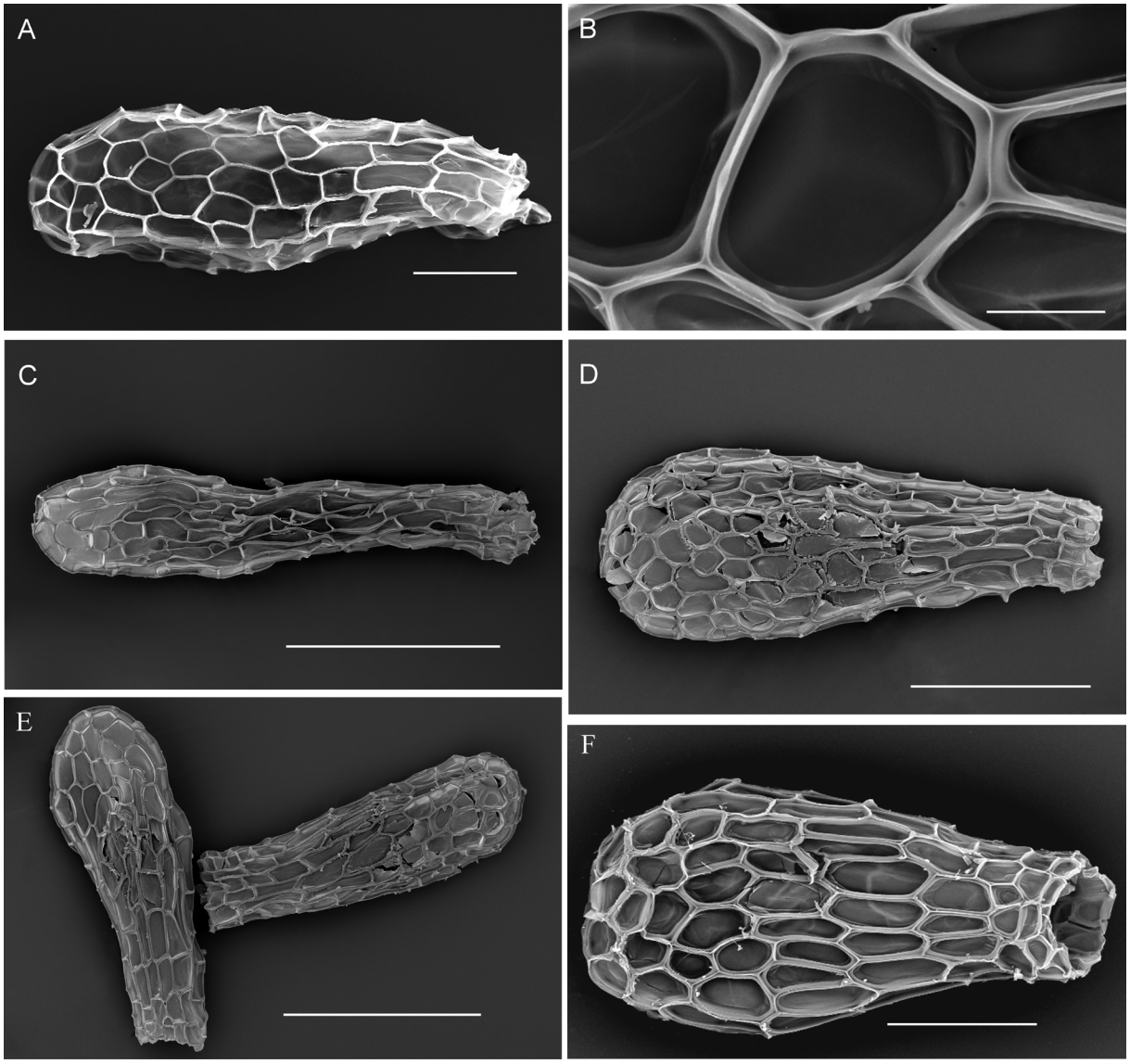

Figure 1. Seed micromorphology. *Dactylorhiza incarnata*: A- seed shape. B- cells of the apical pole. *Dactylorhiza viridis*: C- seed shape. D- seed morphology. E- seed shape. F- *seed morphology*. Scale bars: A and F = 100 μm, B = 20 μm, C and E = 300 μm, D = 200 μm.

and perpendicular to the longitudinal axis (Figure 3C and 3D), and curved. In this group, all the samples analyzed show fusiform shape (Figure 3E) with the embryo in a central position and raised lamella (Figure 3F). For the same taxon, we found a wide variability in the ornamentation pattern among populations, as we can see in *D. maculata* subsp. *maculata* and *D. fuchsii*, although in this species the transversal trabeculae are more frequent. This type of ornamentation appears in the taxa of the *maculata* group (*D. foliosa*, *D. fuchsii*, *D. maculata* subsp. *maculata*, *D. maculata* subsp. *caramulensis*) and the *majalis* group, which includes the taxa *D. alpestris*, *D. cordigera*, *D. majalis* subsp. *majalis*, *D. purpurella*, and *D. elata* (Figure 4A, 4B, and 4C). In the *majalis* group, the principal difference is the presence of curved to sinuous anticlinal walls along the cell in *D. alpestris*. As in the rest of the taxa, raised lamella in the anticlinal walls can be seen (Figure 4D).

In the genus *Gymnadenia*, the seeds are clavate, with smooth periclinal walls (Figure 5A). Within the genus, strong variability in the shape of the anticlinal walls was found, from straight (Figure 5B) to sinuous or wavy (Figure 5C), including all the intermediate grades of curvature (Figures 5D and 5E). Along the longitudinal axis, medial to basal cells are longer than the rest (Figure 5F).

Pseudorchis show fusiform seeds, with the widest part in the central region (Figure 6A). All the cells are similar

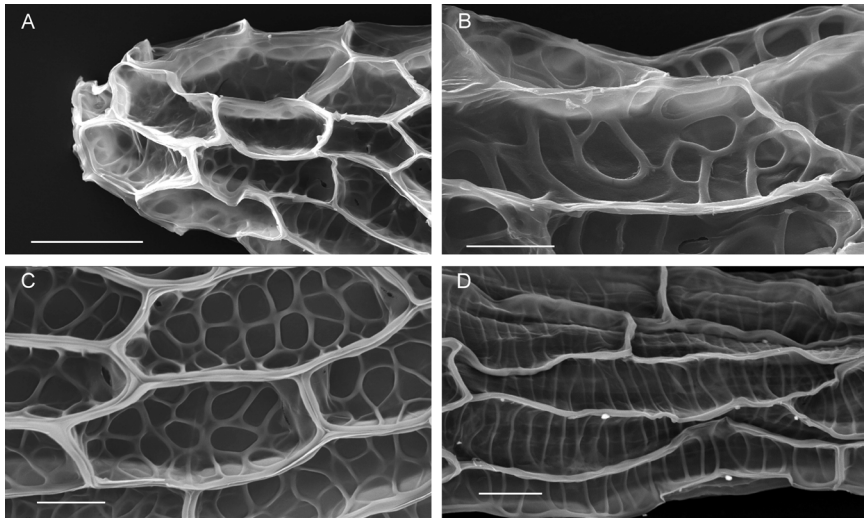

Figure 2. Seed micromorphology. *Dactylorhiza sambucina*: A- apical pole. B- ornamentation of the medial cells. *Dactylorhiza romana* subsp. *guimaraesii*: C- ornamentation of the periclinal and anticlinal walls. *Dactylorhiza insularis*: D- ornamentation of the periclinal walls. Scale bars: A = 100 µm, B, C and D = 20 µm.

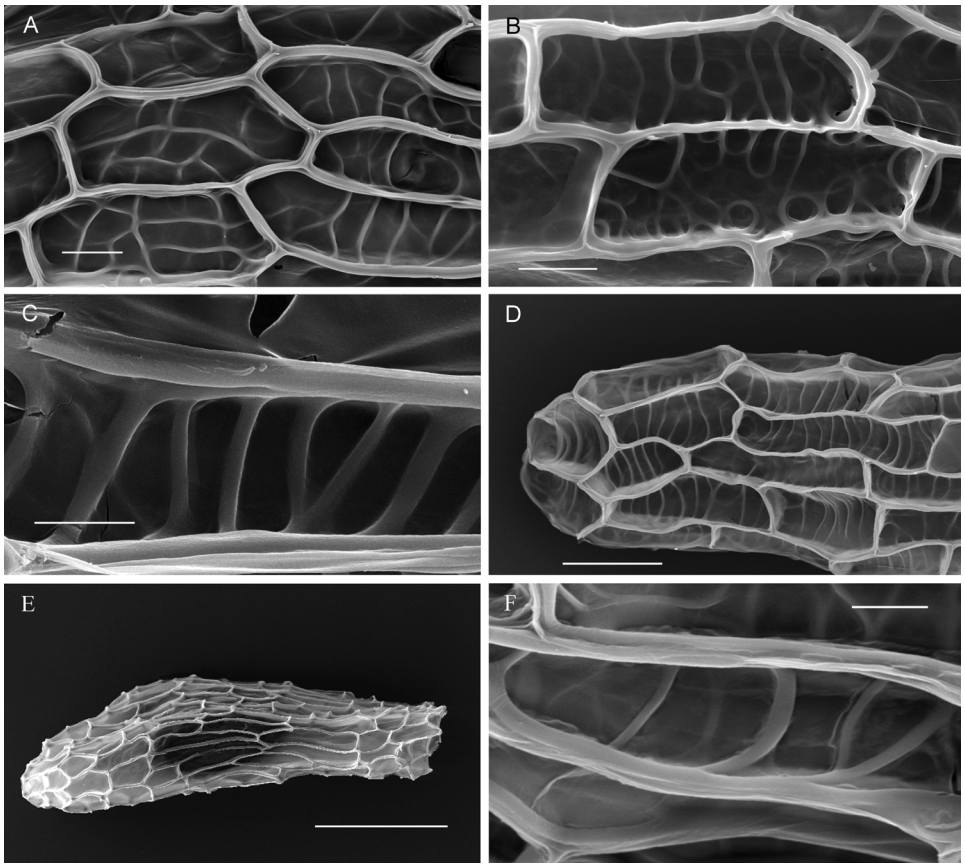

Figure 3. Seed micromorphology. *Dactylorhiza maculata* subsp. *maculata*: A- ornamentation of the medial cells, B- ornamentation of the periclinal walls. *Dactylorhiza fuchsii*: C- ornamentation of the medial cells, D- apical pole and ornamentation of the periclinal walls. *Dactylorhiza foliosa*: E- seed shape. *Dactylorhiza maculata* subsp. *caramulensis*: F- detail of the ornamentation of the periclinal and anticlinal walls. Scale bars: A and B = 20 µm, C and F = 10 µm, D = 50 µm, E = 200 µm.

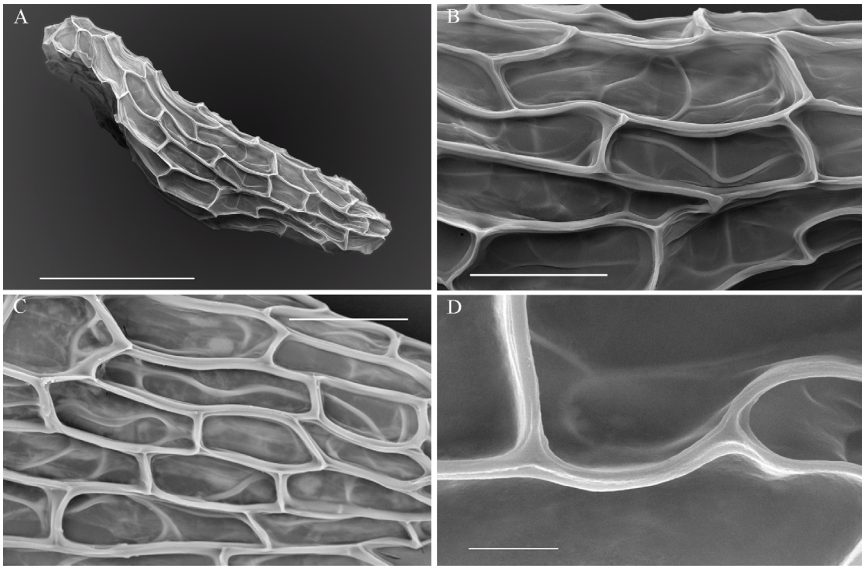

Figure 4. Seed micromorphology. *Dactylorhiza elata*: A- seed shape. B- ornamentation of the periclinal anticlinal walls. *Dactylorhiza majalis* subsp. *majalis*: C- ornamentation of the medial cells. *Dactylorhiza purpurella*: D- anticlinal walls. Scale bars: A = 200 µm, B and C = 50 µm, D = 10 µm.

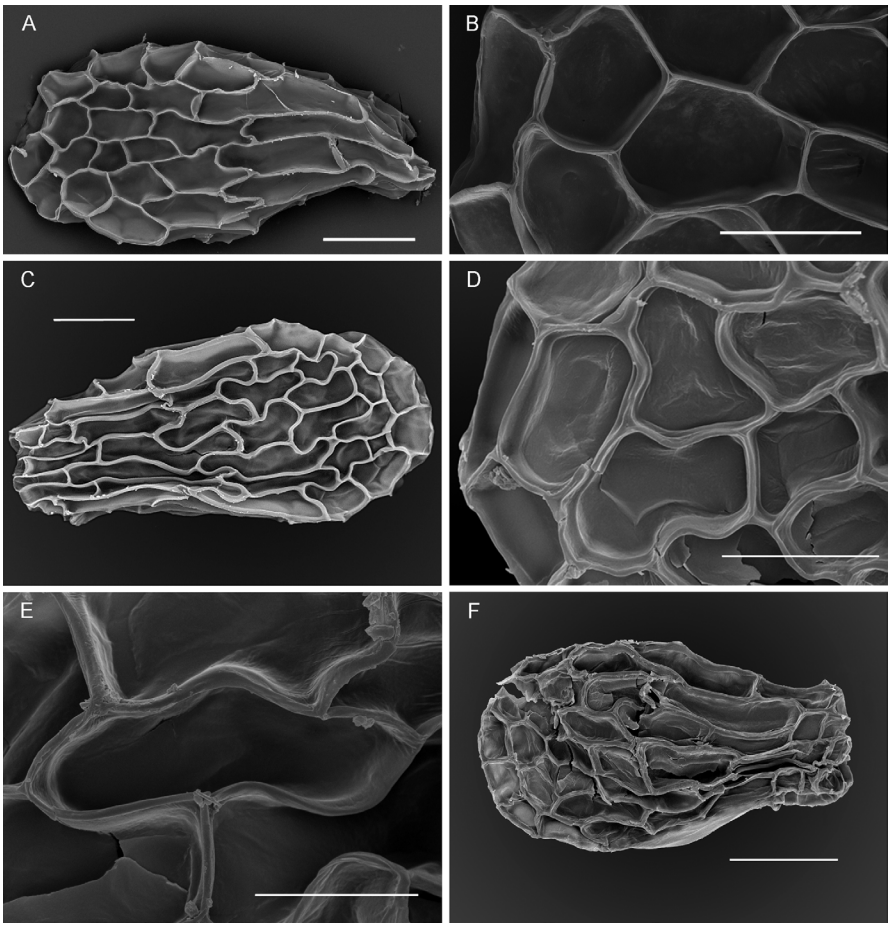

Figure 5. Seed micromorphology. *Gymnadenia odoratissima*: A- seed shape. *Gymnadenia conopsea*: B- apical cells. *Gymnadenia austriaca* var. *gallica*: C- seed shape. *Gymnadenia austriaca*: D- apical pole. *Gymnadenia nigra*: E- apical cell. *Gymnadenia corneliana*: F- seed shape. Scale bars: A and F = 100 µm, B and C = 50 µm, D = 30 µm, E = 20 µm.

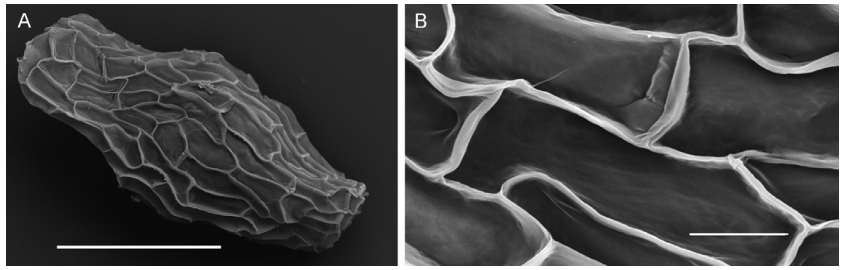

Figure 6. Seed micromorphology. *Pseudorchis albida*: A- seed shape. B- anticlinal and periclinal walls Scale bars: A = 200 μm, B = 20 μm.

along the longitudinal axis, but the medial cells are slightly longer than the basal and apical cells. The anticlinal walls are curved to sinuous, with fine lamella. Periclinal walls without ornamentation (Figure 6B).

In Table 2, the quantitative data show, for each population, the means and standard deviations of length and width of the seeds and embryos, the ratio length/width of the seed, and the number of cells along the longitudinal axis.

Within the genus *Dactylorhiza*, the statistical analysis reflects that the length of the seed is the main value ($P = 0.006$), according to ANOVA for level of significance $P < 0.05$. The analysis of the average values of the populations shows a strong variability in the length (Figure 7), even for the same species, as can be seen in the quantitative data for *D. fuchsii* (populations 23 to 25 in Figure 7) and *D. maculata* subsp. *maculata* (populations 26 to 31 in Figure 7). Despite the differences among these values, the seed shape of these taxa is always fusiform. However, in *D. viridis* we saw a strong concordance between the quantitative data and the seed shape, with low values in the clavate seeds (southern European populations) and high values in the fusiform seeds (North American populations); in the Russian population (Blinova 68a) we found fusiform to clavate seeds, with a high diversity of values as can be seen in Figure 8.

Except for these samples with clavate seeds in *D. viridis* (populations 1A and 1B in Figure 9), the quantitative data of those with fusiform seeds (populations 1C to 1G in Figure 9) are similar to those found in *D. incarnata* (populations 2A to 2F in Figure 9), with which it shares the largest number of qualitative characters within the genus. No significant differences were found for seed length between both species.

Average value of seed length in *Pseudorchis* is similar to those of *Gymnadenia conopsea*, *G. densiflora*, and *G. odoratissima* (Figure 10), but morphologically the shape is fusiform in *Pseudorchis* and clavate in *Gymnadenia*. The rest of the taxa analyzed in this genus show lower values, except for *G. austriaca* var. *gallica*, placed in an intermediate position (Figure 10).

Table 2. Quantitative data of the analyzed populations.

Species	Seed length (μm) ± SD	Seed width (μm) ± SD	Ratio L/W ± SD	Number of cells	Embryo length (μm) ± SD	Embryo width (μm) ± SD
Dactylorhiza viridis (MA 328766)	443.35 ± 50.73	164.04 ± 23.85	2.73 ± 0.30	7–10	148.70 ± 23.20	105.85 ± 17.23
D. viridis (MAF 37102)	319.00 ± 46.75	179.22 ± 30.80	1.79 ± 0.25	7–10	176.59 ± 38.23	132.65 ± 28.95
D. viridis (Blinova 68a)	495.64 ± 68.35	208.12 ± 29.21	2.40 ± 0.33	7–11	184.26 ± 18.24	146.57 ± 14.77
D. viridis (US 648754)	614.92 ± 50.17	162.77 ± 22.55	3.83 ± 0.50	9–13	151.99 ± 20.85	108.18 ± 9.03
D. viridis (US 1366192)	516.94 ± 65.78	176.57 ± 24.12	2.96 ± 0.44	7–12	146.72 ± 16.44	100.57 ± 13.16
D. viridis (US 3021875)	596.59 ± 52.96	155.04 ± 19.09	3.90 ± 0.59	8–11	158.86 ± 10.97	104.66 ± 8.33
D. viridis (US 3614760)	559.00 ± 62.91	160.75 ± 23.00	3.54 ± 0.61	8–12	157.20 ± 16.83	108.97 ± 7.31
D. alpestris (Gamarra 198)	558.31 ± 52.16	153.69 ± 29.56	3.76 ± 0.78	9–12	214.63 ± 27.92	147.81 ± 20.42
D. elata (Gamarra 334)	653.27 ± 90.31	215.51 ± 31.95	3.08 ± 0.52	7–11	276.44 ± 32.31	185.13 ± 22.34
D. elata (Gamarra 419)	583.94 ± 47.95	191.82 ± 25.18	3.08 ± 0.39	9–14	199.07 ± 14.74	122.71 ± 13.01
D. elata (Gamarra 439)	647.00 ± 78.61	185.25 ± 15.85	3.49 ± 0.34	8–11	192.34 ± 21.95	120.70 ± 16.68
D. foliosa (Dalgaard 13124)	592.98 ± 40.92	246.48 ± 29.77	2.44 ± 0.33	8–10	259.19 ± 29.94	179.72 ± 24.08
D. incarnata (MAF 118725)	613.75 ± 62.82	196.25 ± 17.91	3.14 ± 0.39	8–10	184.47 ± 22.84	140.71 ± 20.98
D. incarnata (Blinova 62)	574.94 ± 49.36	304.48 ± 41.62	1.92 ± 0.30	9–12	188.83 ± 15.68	137.67 ± 13.51
D. incarnata (Gamarra 623)	555.87 ± 49.32	261.81 ± 32.97	2.16 ± 0.34	10–13	182.72 ± 15.47	122.03 ± 11.62

Table 2. (Continued).

Species	Seed length (µm) ± SD	Seed width (µm) ± SD	Ratio L/W ± SD	Number of cells	Embryo length (µm) ± SD	Embryo width (µm) ± SD
D. incarnata (Pedersen 778)	659.38 ± 76.99	226.73 ± 32.86	2.97 ± 0.54	8–12	189.39 ± 19.81	142.59 ± 16.85
D. incarnata (Galán 4372)	647.27 ± 37.35	190.00 ± 17.85	3.43 ± 0.40	10–13	194.36 ± 10.21	127.36 ± 9.12
D. incarnata (Galán 4378)	733.33 ± 65.44	265.65 ± 33.04	2.80 ± 0.39	12–16	231.95 ± 15.61	160.81 ± 15.41
D. insularis (Pedersen)	520.80 ± 66.44	152.96 ± 18.98	3.43 ± 0.40	5–9	161.06 ± 25.35	108.30 ± 16.69
D. insularis (Gamarra 280)	510.07 ± 57.63	143.74 ± 14.66	3.59 ± 0.57	6–8	155.94 ± 23.13	113.95 ± 12.77
D. insularis (Galán 4474)	439.14 ± 50.30	135.65 ± 13.63	3.26 ± 0.43	6–8	152.48 ± 21.98	111.91 ± 16.79
D. maculata subsp. *caramulensis* (Galán 4455)	833.81 ± 47.19	239.24 ± 24.94	3.52 ± 0.52	8–11	251.38 ± 42.63	183.71 ± 26.55
D. fuchsii (Gamarra 317)	590.21 ± 64.11	164.08 ± 26.91	3.69 ± 0.68	8–13	167.35 ± 29.31	110.01 ± 20.55
D. fuchsii (Gamarra 169)	791.93 ± 61.56	153.87 ± 25.39	5.27 ± 0.90	8–12	184.57 ± 21.13	121.49 ± 13.39
D. fuchsii (Wiinstedt)	1037.06 ± 78.48	232.86 ± 28.83	4.52 ± 0.66	9–12	226.50 ± 24.66	157.78 ± 23.67
D. maculata subsp. *maculata* (Gamarra 215)	415.00 ± 45.51	113.89 ± 13.37	3.66 ± 0.36	7–10	146.11 ± 18.75	94.00 ± 13.41
D. maculata subsp. *maculata* (Gamarra 455)	766.94 ± 69.17	192.04 ± 19.58	4.02 ± 0.42	8–11	200.78 ± 18.41	122.08 ± 14.75
D. maculata subsp. *maculata* (Blinova 7)	819.39 ± 95.91	265.02 ± 40.20	3.14 ± 0.48	7–10	235.51 ± 33.31	168.97 ± 32.62
D. maculata subsp. *maculata* (Gamarra 335)	689.17 ± 80.40	199.44 ± 31.62	3.53 ± 0.62	8–11	272.33 ± 29.74	168.15 ± 20.02
D. maculata subsp. *maculata* (Galán 4371)	712.72 ± 69.99	237.52 ± 30.04	3.04 ± 0.46	7–10	216.58 ± 22.02	134.94 ± 21.81
D. cordigera (Strid 16341)	727.27 ± 64.87	261.67 ± 33.26	2.82 ± 0.42	9–12	221.49 ± 23.87	143.07 ± 18.97
D. majalis (Gamarra 217)	723.42 ± 84.75	188.32 ± 23.32	3.89 ± 0.64	9–12	195.42 ± 20.01	144.87 ± 17.02
D. majalis (Hansen)	825.56 ± 68.40	212.58 ± 29.91	3.95 ± 0.59	9–12	231.88 ± 21.72	159.88 ± 18.39
D. majalis (Gamarra 320)	536.86 ± 51.82	206.21 ± 26.47	2.65 ± 0.47	8–11	174.23 ± 21.67	123.85 ± 17.06
D. majalis (Gamarra 458)	697.43 ± 50.95	210.00 ± 22.62	3.36 ± 0.46	10–12	229.75 ± 22.59	122.03 ± 15.89
D. purpurella (Dahl)	738.07 ± 77.74	175.12 ± 18.43	4.25 ± 0.61	10–12	180.34 ± 15.69	124.67 ± 14.61
D. romana subsp. *romana* (Pedersen 776)	634.54 ± 68.54	185.21 ± 23.54	3.48 ± 0.57	6–8	168.99 ± 15.36	112.13 ± 11.17
D. sambucina (Gamarra 663)	552.44 ± 65.50	203.70 ± 24.81	2.73 ± 0.35	6–8	186.01 ± 22.63	126.32 ± 15.20
D. sambucina (Gamarra 331a)	615.08 ± 51.60	207.15 ± 25.07	3.00 ± 0.36	6–9	236.47 ± 26.94	151.42 ± 16.57
D. sambucina (Gamarra 331b)	572.05 ± 73.74	188.46 ± 19.80	3.06 ± 0.50	6–9	224.79 ± 33.71	159.39 ± 28.31
D. sambucina (Gamarra 618)	490.36 ± 32.28	158.37 ± 24.10	3.16 ± 0.49	6–9	152.12 ± 15.48	106.35 ± 12.20
D. sambucina (Hartvig 7168)	629.38 ± 49.19	192.28 ± 20.50	3.30 ± 0.41	6–10	191.47 ± 26.21	135.56 ± 17.92
D. romana subsp. *guimaraesii* (Gamarra 608)	732.86 ± 85.66	196.90 ± 27.47	3.77 ± 0.57	6–10	163.40 ± 21.50	113.33 ± 17.92
D. romana subsp. *guimaraesii* (Galán 4403)	764.99 ± 75.55	188.40 ± 19.70	4.09 ± 0.47	7–10	177.10 ± 22.71	117.70 ± 18.26
Gymnadenia conopsea (Blinova 65)	398.24 ± 43.35	220.05 ± 22.22	1.83 ± 0.27	5–7	189.42 ± 27.73	143.57 ± 29.26
G. conopsea (Gamarra 324)	432.37 ± 50.63	185.08 ± 19.15	2.35 ± 0.31	5–9	183.84 ± 15.50	120.43 ± 14.51
G. conopsea (Gamarra 406)	430.52 ± 36.98	173.16 ± 16.12	2.51 ± 0.32	7–9	205.82 ± 17.73	133.45 ± 16.30
G. conopsea (Gamarra 453)	479.74 ± 46.87	167.69 ± 16.46	2.89 ± 0.43	7–9	174.94 ± 21.67	109.82 ± 19.59
G. conopsea (Galán 4461)	519.69 ± 62.57	234.31 ± 28.84	2.23 ± 0.38	6–8	181.47 ± 24.28	139.15 ± 20.46
G. conopsea (MAF 36725)	429.01 ± 54.05	193.79 ± 27.92	2.27 ± 0.48	5–8	171.27 ± 23.46	111.11 ± 14.92
G. densiflora (W 1994: 03286)	452.56 ± 44.49	151.47 ± 18.45	3.03 ± 0.45	7–8	158.20 ± 18.05	103.8 ± 13.97
G. odoratissima (W 1978: 13087)	425.83 ± 38.30	202.02 ± 28.07	2.15 ± 0.39	5–8	181.40 ± 24.77	136.36 ± 17.73
G. odoratissima (W 1968: 18743)	435.16 ± 30.75	195.48 ± 21.11	2.25 ± 0.30	7–9	233.05 ± 23.51	171.07 ± 22.47
G. austriaca (Hanak 03/68)	295.93 ± 15.19	153.46 ± 11.23	1.93 ± 0.08	9–11	109.23 ± 6.90	116.76 ± 13.69
G. austriaca var. *gallica* (Galán 4463)	370.89 ± 34.03	173.78 ± 17.75	2.16 ± 0.31	5–7	183.35 ± 14.48	116.76 ± 13.69
G. austriaca var. *gallica* (Gamarra 326)	394.00 ± 31.03	187.33 ± 16.59	2.12 ± 0.26	5–7	190.61 ± 18.38	133.81 ± 16.72
G. corneliana (Palisieux)	275.69 ± 13.54	160.78 ± 6.72	1.71 ± 0.07	7–9	126.91 ± 6.57	84.79 ± 4.59
G. miniata (Roessler 629)	304.01 ± 10.16	193.24 ± 8.53	1.57 ± 0.08	8–10	165.18 ± 7.31	101.96 ± 6.70
G. nigra (Podlech 24526)	297.70 ± 15.02	180.06 ± 11.64	1.65 ± 0.08	7–9	130.38 ± 12.84	85.61 ± 6.69
Pseudorchis albida (Gamarra 329)	436.5 ± 45.77	180.75 ± 20.43	2.45 ± 0.42	7–9	179.00 ± 24.50	140.04 ± 25.22

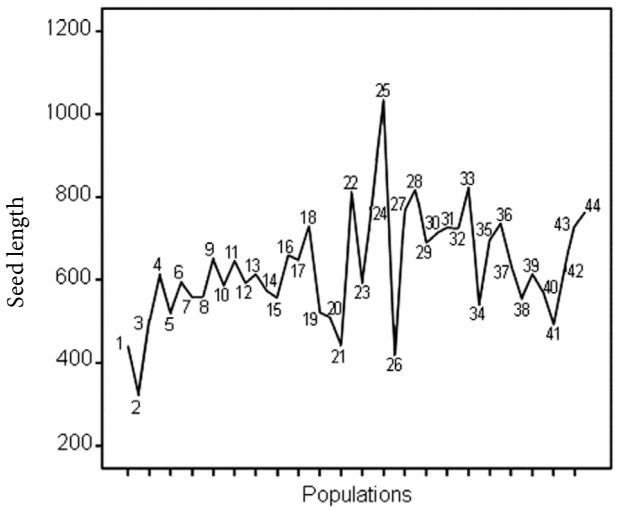

Figure 7. Average values of seed length in the populations analyzed of the genus *Dactylorhiza*. 1–7: *D. viridis*; 8: *D. alpestris*; 9–11: *D. elata*; 12: *D. foliosa*; 13–18: *D. incarnata*; 19–21: *D. insularis*; 22: *D. maculata* subsp. *caramulensis*; 23–25: *D. fuchsii*; 26–30: *D. maculata* subsp. *maculata*; 31: *D. cordigera*; 32–35: *D. majalis*; 36: *D. purpurella*; 37: *D. romana* subsp. *romana*; 38–42: *D. sambucina*; 43–44: *D. romana* subsp. *guimaraesii*.

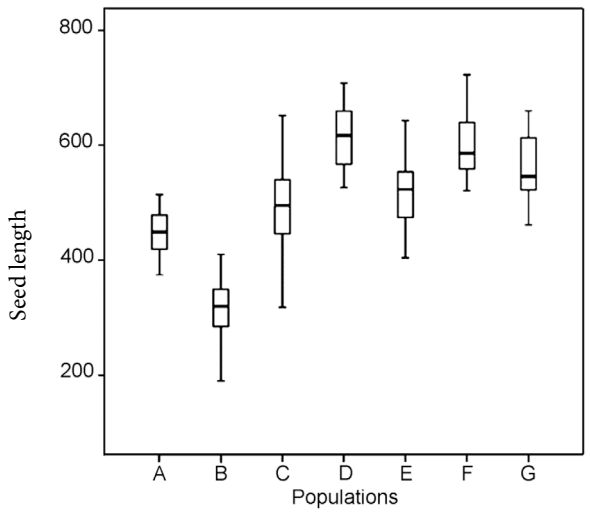

Figure 8. Average values of seed length in the populations analyzed of *Dactylorhiza viridis*. Samples A and B: clavate seeds; C: fusiform to clavate seeds. D–G: fusiform seeds. A: MA 328766. B: MAF 37102. C: Blinova 68a. D: US 648754. E: US 1366192. F: US 3021875. G: US 3614760.

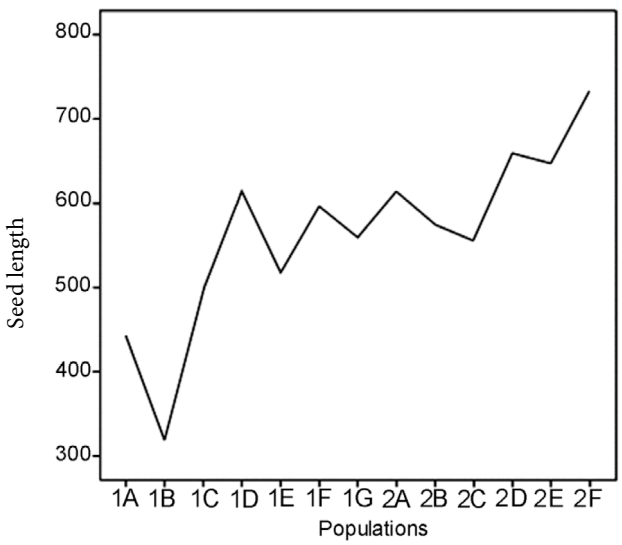

Figure 9. Average values of seed length in the populations analyzed of *Dactylorhiza viridis* (1) and *D. incarnata* (2). 1A: MA 328766. 1B: MAF 37102. 1C: Blinova 68a. 1D: US 648754. 1E: US 1366192. 1F: 3021875. 1G: 3614760. 2A: MAF 118725. 2B: Blinova 62. 2C: Gamarra 623. 2D: Pedersen 778. 2E: Galán 4372. 2F: Galán 4378.

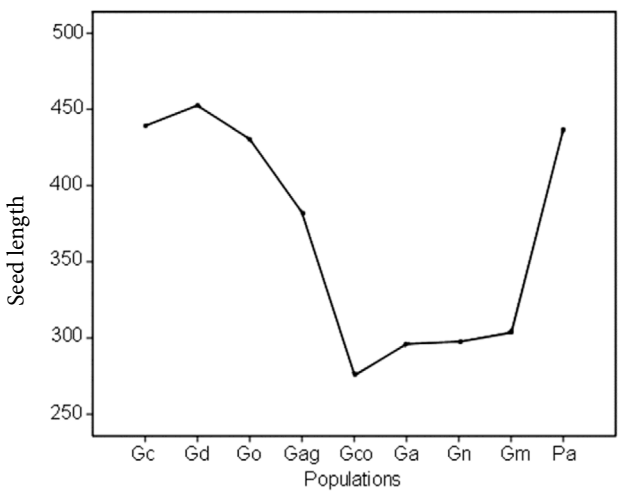

Figure 10. Average values of seed length in the taxa analyzed of *Gymnadenia* and *Pseudorchis*. Gc: *G. conopsea*; Gd: *G. densiflora*; Go: *G. odoratissima*; Gag: *G. austriaca* var. *gallica*; Gco: *G. corneliana*; Ga: *G. austriaca*; Gn: *G. nigra*; Gm: *G. miniata*; Pa: *Pseudorchis albida*.

4. Discussion

In our previous studies about the seed micromorphology in some genera of the tribe Orchideae, we corroborated the taxonomic value of certain qualitative and quantitative characters (Gamarra et al., 2007, 2008, 2010, 2012). These results are in concordance with earlier cytogenetic (Cauwet-Marc and Balayer, 1984), morphological (Kretzschmar et al., 2007), and molecular data (Pridgeon et al., 1997; Bateman et al., 2003).

Within the genus *Dactylorhiza*, the fusiform shape and the raised lamella in the anticlinal walls are qualitative characters common to all taxa. We delimited 3 groups according to the ornamentation of the periclinal walls.

In the *incarnata* group, the cells are smooth, a feature previously observed by Averyanov (1990). Our recognition of this group is congruent with morphometric (Tyteca and Gathoye, 1989) and molecular data (Hedrén, 1996, 2001; Hedrén et al., 2001; Pillon et al., 2007). We found similar seeds in the North American populations of *D. viridis* (= *Coeloglossum viride*). This species shows a tendency for decreased measures of seed length in Eurasia, and mainly in southern Europe agrees with the change in seed morphology to clavate seeds. Our results support the inclusion of the former monotypic genus *Coeloglossum* in *Dactylorhiza*, as corroborated by molecular analyses (Bateman et al., 2003).

A second group comprises the species included in the so-called *sambucina* group (Delforge, 2006). The seeds showed reticulated testa cells, as previously observed by Averyanov (1990). The trabeculae are anastomosed in the diploid species *D. sambucina*, *D. romana* subsp. *romana*, and *D. romana* subsp. *guimaraesii*. However, in the triploid species *D. insularis*, with a hybrid origin (Bullini et al., 2001; Pedersen, 2006), the trabeculae are mainly transversal to the longitudinal axis. This pattern of ornamentation has been also observed in *D. cantabrica*, but we need to analyze more seeds and more populations to confirm it. This group is morphologically distinct from the rest of the genus (Tyteca and Gathoye, 1989), but conflicting data exist as to whether or not it is monophyletic (Hedrén et al., 2001; Pillon et al., 2007).

The taxa of the *maculata* group (*D. maculata* subsp. *maculata*, *D. maculata* subsp. *caramulensis*, *D. fuchsii*, *D. foliosa*) showed a wide variability in the ornamentation of the periclinal walls. Only in *D. fuchsii* is the ornamentation pattern more homogeneous, predominantly with transversal trabeculae. Probably, this feature is in concordance with the mainly diploid nature of this taxon, which appears closely related to an unknown (possibly extinct) ancestor of other taxa of this group (Pillon et al., 2007; Ståhlberg and Hedrén, 2010). Our recognition of the *D. maculata* group is congruent with morphometric (Tyteca and Gathoye, 1989) and molecular data (Hedrén, 1996; Hedrén et al., 2001; Pillon et al., 2007).

As demonstrated by several authors (Hedrén, 1996; Shipunov et al., 2004; Hedrén et al., 2007; Pillon et al., 2007), taxa of the *maculata* and *incarnata* groups, through allopolyploidization, have generated the vast majority of taxa recognized in the so-called *majalis*, *traunsteineri*, and *praetermissa* groups sensu Delforge (2006). The great morphological, cytological, and ecological diversity of all these groups, as indicated by the different authors mentioned above, is corroborated by the higher degree of variability in sculpturing of the periclinal walls.

In the allopolyploids *D. alpestris*, *D. elata*, and *D. majalis*, we observed variability in the ornamentation, with transversal, anastomosed, or curved trabeculae, oriented longitudinally to the major axis of the seeds. In contrast to our observations in the *incarnata* group, and in other genera of the tribe Orchideae (Gamarra et al., 2007, 2008, 2012), we did not find a homogeneous pattern in the seeds of one and the same taxon, and quantitative data show strong differences between populations of the same species. This is hardly surprising, given the recurrent origins of several allotetraploid taxa of *D. incarnata* s.l. × *maculata* s.l. parentage and the various degrees of introgression that some populations have experienced (Pillon et al., 2007; Hedrén et al., 2011).

In *Gymnadenia* we found clavate seeds without ornamentation in the periclinal walls and more or less raised lamella in the stout anticlinal walls. The shape of the anticlinal walls shows strong variability among the species, from straight to wavy. Our results are similar to those published by Teppner and Klein (1994) and Sáez (2004), and, likewise, we did not find ornamentation in the periclinal walls as Haas (1977) reported for *Nigritella nigra*. Quantitative data show 2 groups of seeds related to the length, corresponding to the minor values with the taxa previously included in the genus *Nigritella*, but the number of populations analyzed is low, and it is necessary to study in the future more populations to elucidate the taxonomic position of these genera.

The fusiform seeds with fine anticlinal walls of *Pseudorchis* are clearly distinguished from the clavate seeds with stout anticlinal walls of *Gymnadenia*, although both share features such as the absence of sculpture in the periclinal walls and similar average values of seed length. We agree with the taxonomic view of Reinhammar (1998), which is supported by the molecular phylogenetic analysis of Bateman et al. (2003), that *Pseudorchis* should be maintained as an independent genus, and not embedded into *Gymnadenia* as proposed by Delforge (2006).

Our study emphasizes the strong support between the seed micromorphology and the molecular analyses published by different authors, supporting the inclusion of *Coeloglossum* within *Dactylorhiza*, and confirming the

independent status of the genus *Pseudorchis*. In the genus *Dactylorhiza*, the *incarnata* and *sambucina* groups show their own pattern in the periclinal walls of the testa cells. The variability in the ornamentation on the seeds of the taxa belonging to the *majalis*, *maculata*, and *praetermissa* groups of the genus *Dactylorhiza* is congruent with the genetic processes that occurred during the history of this genus.

Acknowledgments

This investigation was carried out within the framework of a research project supported by the Universidad Autónoma de Madrid (UAM). Technical assistance from the staff (Esperanza Salvador, Enrique Rodríguez, Isidoro Poveda, and Yolanda Ruiz) in the SEM laboratories at SIDI-UAM and RJB-CSIC is gratefully acknowledged. The authors express their gratitude to the curators and collection managers of the herbaria C, M, MA, MAF, and W for the loan of material.

References

Aedo C, Herrero A (2005). Orchidaceae. In: Castroviejo S et al., editors. Flora Iberica. Vol. XXI. Madrid, Spain: CSIC, pp. 15–97 (in Spanish).

Amich F, García-Barriuso M, Bernardos S (2007). Polyploidy and speciation in the orchid flora of the Iberian Peninsula. Bot Helv 117: 143–157.

Arditti J, Michaud, JD, Healey PL (1979). Morphometry of orchid seeds. I. *Paphiopedilum* and native California and related species of *Cypripedium*. Am J Bot 66: 1128–1137.

Averyanov LV (1990). A review of the genus *Dactylorhiza*. In: J Arditti, editor. Orchid Biology. Reviews and Perspectives, V. Portland, OR, USA: Timber Press, pp. 159–206.

Barthlott W (1976). Morphologie der Samen von Orchideen im Hinblick auf taxonomische und funktionelle Aspekte. In: K Senghas, editor. Proceedings of the 8th World Orchid Conference Frankfurt. Hamburg, Germany: Parey, pp. 444–455 (in German).

Barthlott W, Ziegler B (1981). Mikromorphologie der Samenschalen als systematisches Merkmal bei Orchideen. Ber Deut Bot Ges 94: 267–273 (in German).

Bateman RM (2009). Evolutionary classification of European orchids: the crucial importance of maximising explicit evidence and minimising authoritarian speculation. J Eur Orch 41: 243–318.

Bateman RM, Hollingsworth PM, Preston J, Yi-bo L, Pridgeon AM, Chase MW (2003). Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidadeae). Bot J Linn Soc 142: 1–40.

Bullini L, Cianchi R, Arduino P, De Bonis L, Mosco MC, Verardi A, Porretta D, Corrias B, Rossi W (2001). Molecular evidence for allopolyploid speciation and a single origin of the western Mediterranen orchid *Dactylorhiza insularis* (Orchidaceae). Biol J Linn Soc 72: 193–201.

Cauwet-Marc AM, Balayer M (1984). Les genres *Orchis* L., *Dactylorhiza* Necker ex Nevski, *Neotinea* Reichb. et *Traunsteinera* Reichb.: Caryologie et proposition de phylogènie et d'évolution. Bot Helv 94: 391–406 (in French).

Chase MW, Pippen JS (1988). Seed morphology in the Onciidinae and related subtribes (Orchidaceae). Syst Bot 13: 313–323.

Clifford HT, Smith WK (1969). Seed morphology and classification of Orchidaceae. Phytomorphology 19: 133–139.

Delforge P (2006). Orchids of Europe, North Africa and the Middle East. 3rd ed. London, UK: A&C Black.

Devos N, Raspé O, Jacquemart A-L, Tyteca D (2006). On the monophyly of *Dactylorhiza* Necker ex Nevski (Orchidaceae): is *Coeloglossum viride* (L.) Hartman a *Dactylorhiza*? Bot J Linn Soc 152: 261–269.

Gamarra R, Dorda E, Scrugli A, Galán P, Ortúñez E (2007). Seed micromorphology in the genus *Neotinea* Rchb.f. (Orchidaceae, Orchidinae). Bot J Linn Soc 153: 133–140.

Gamarra R, Galán P, Herrera I, Ortúñez E (2008). Seed micromorphology supports the splitting of *Limnorchis* from *Platanthera* (Orchidaceae). Nord J Bot 26: 61–65.

Gamarra R, Ortúñez E, Galán Cela P, Guadaño V (2012). *Anacamptis* versus *Orchis* (Orchidaceae): seed micromorphology and its taxonomic significance. Plant Syst Evol 298: 597–607.

Gamarra R, Ortúñez E, Sanz E, Esparza I, Galán P (2010). Seeds in subtribe Orchidinae (Orchidaceae): the best morphological tool to support molecular analyses. In: Nimis PL, Vignes Lebbe R, editors. Tools for identifying Biodiversity: Progress and Problems. Trieste, Italy: Edizioni Università di Trieste, pp. 323–326.

Govaerts R, Bernet P, Kratochvil K, Gerlach G, Carr G, Alrich P, Pridgeon AM, Pfahl J, Campacci MA, Holland Baptista D et al. (2014). World Checklist of Selected Plant Families. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet; http://apps.kew.org/wcsp/ [accessed 09 July 2014].

Haas HF (1977). Asymbiontische Vermehrung europäischer Erdorchideen. II. *Nigritella nigra* (L.) Rchb. f. und *Nigritella miniata* (CR) Jamchen. Die Orchidee 28: 69–73 (in German).

Hedrén M (1996). Genetic differentiation, polyploidization and hybridization in northern European *Dactylorhiza* (Orchidaceae): evidence from allozyme markers. Plant Syst Evol 201: 31–55.

Hedrén M (2001). Systematics of the *Dactylorhiza euxina*/*incarnata*/*maculata* polyploid complex (Orchidaceae) in Turkey: evidence from allozyme data. Plant Syst Evol 229: 23–44.

Hedrén M, Fay MF, Chase MW (2001). Amplified fragment length polymorphism (AFLP) reveal details of polyploid evolution in *Dactylorhiza* (Orchidaceae). Am J Bot 88: 1868–1880.

Hedrén M, Klein E, Teppner H (2000). Evolution of polyploids in the European orchid genus *Nigritella*: evidence from allozyme data. Phyton-Ann Rei Bot A 40: 239–275.

Hedrén M, Nordström S, Bateman RM (2011). Plastic and nuclear DNA marker data support the recognition of four tetraploid marsh orchids (*Dactylorhiza majalis s.l.*, Orchidaceae) in Britain and Ireland, but require their recircumscription. Biol J Linn Soc 104: 107–128.

Hedrén M, Nordström S, Persson Hovmalm HA, Pedersen HÆ, Hansson S (2007). Patterns of polyploid evolution in Greek marsh orchids (*Dactylorhiza*: Orchidaceae) as revealed by allozymes, AFLPs, and plastid DNA data. Am J Bot. 94: 1205–1218.

Kretzschmar H, Eccarius W, Dietrich H (2007). The Orchid Genera *Anacamptis*, *Orchis*, *Neotinea*. Bürgel, Germany: EchinoMedia.

Mrkvicka AC (1994). Anatomie und Morphologie der Samen heimischer Orchideenarten. J Eur Orch 26: 168–314 (in German).

Pedersen HÆ (1998). Species concept and guidelines for infraspecific taxonomic ranking in *Dactylorhiza* (Orchidaceae). Nord J Bot 18: 289–310.

Pedersen HÆ (2006). Systematics and evolution of the *Dactylorhiza romana/sambucina* polyploid complex (Orchidaceae). Bot J Linn Soc 152: 405–434.

Pedersen HÆ (2010). Inadequate morphometric analyses have contributed to oversplitting in European orchids: a case study in *Dactylorhiza* (Orchidaceae). In: O Seberg, G Petersen, AS Barfod, JI Davis, editors. Diversity, phylogeny, and evolution in the monocotyledons. Proceedings of the Fourth International Conference on the Comparative Biology of the Monocotyledons and the Fifth International Symposium on Grass Systematics and Evolution. Aarhus, Denmark: Aarhus University Press, pp. 193–212.

Pillon Y, Fay MF, Hedrén M, Bateman RM, Devey DS, Shipunov AB, van der Bank M, Chase MW (2007). Evolution and temporal diversification of western European polyploid species complexes in *Dactylorhiza* (Orchidaceae). Taxon 56: 1185–1208.

Pillon Y, Fay MF, Shipunov AB, Chase MW (2006). Species diversity versus phylogenetic diversity: a practical study in the taxonomically difficult genus *Dactylorhiza* (Orchidaceae). Biol Conserv 129: 4–13.

Pridgeon AM, Bateman RM, Cox AV, Hapeman JR, Chase MW (1997). Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 1. Intergeneric relationships and polyphyly of *Orchis* sensu lato. Lindleyana 12: 89–109.

Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN (2001). Genera Orchidacearum. Volume 2. Orchidoideae (part one). Oxford, UK: Oxford University Press.

Reinhammar LG (1998). Systematics of *Pseudorchis albida* s.l. (Orchidaceae) in Europe and North America. Bot J Linn Soc 126: 363–382.

Sáez L (2004). The genus *Nigritella* (Orchidaceae) in the Iberian Peninsula. An Jard Bot Madrid 61: 81–90.

Sheviak CJ, Catling PM, Meades SJ, Bateman RM (2002). 28. *Dactylorhiza*. In: Flora of North America Editorial Committee, editors. Flora of North America north of Mexico 26. Magnoliophyta: Liliidae: Liliales and Orchidales., New York, NY, USA: Oxford University Press, pp. 577–579.

Shipunov AB, Fay MF, Pillon Y, Bateman RM, Chase MW (2004). *Dactylorhiza* (Orchidaceae) in European Russia: combined molecular and morphological analysis. Am J Bot 91: 1419–1426.

Ståhlberg D, Hedrén M (2010). Evolutionary history of the *Dactylorhiza maculata* polyploid complex (Orchidaceae). Biol J Linn Soc 101: 503–525.

Szlachetko D, Rutkowski P (2000). Gynostemia Orchidalium I. Acta Bot Fenn 169: 1–380.

Teppner H, Klein E (1994). *Nigritella gabasiana* spec. Nova, *N. nigra* subsp. *iberica* subsp. nova (Orchidaceae-Orchideae) und deren Embryologie. Phyton-Ann Rei Bot A 33: 179–209 (in German).

Teppner H, Klein E (1998). Etiam atque etiam – *Nigritella* versus *Gymnadenia*: Neukombinationen und *Gymnadenia dolomitensis* spec. nova (Orchidaceae-Orchideae). Phyton-Ann Rei Bot A 38: 220–224 (in German).

Thiers B (2012). Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. http://sweetgum.nybg.org/ih/

Tohda H (1983). Seed morphology in Orchidaceae I. *Dactylorchis, Orchis, Ponerorchis, Chondradenia* and *Galeorchis*. Sci Rep Res Tohoku, 4th ser, Biol 38: 253–268.

Tsutsumi C, Yukawa T, Sook Lee N, Shook Lee C, Kato M (2007). Phylogeny and comparative seed morphology or epiphytic and terrestrial species of *Liparis* (Orchidaceae) in Japan. J Plant Res 120: 405–412.

Tyteca D, Gathoye JL (1989). Contribution à l'étude biostatistique des *Dactylorhiza* d'Europe occidentale. Mem Soc Roy Bot Belg 11: 43–64 (in French).

Appendix

Taxa are listed in alphabetical order. The information includes the taxon and its authority, locality, collector and collector's number, and the voucher.

Dactylorhiza alpestris (Pugsley) Verm. Andorra: Coll d'Ordino, R. Gamarra 198 (MAUAM). Coll d'Envalira, R. Gamarra 199 (MAUAM).

Dactylorhiza cantabrica H.A. Pedersen. Spain: Lugo, Carbedo, R. Gamarra 603 (MAUAM).

Dactylorhiza cordigera (Fries) Soó. Greece: Nom. Diamas, Elatia, Strid & Papanicolau 16341 (C).

Dactylorhiza elata (Poir.) Soó. Spain: Burgos, Torres de Arriba, P. Galán 4356 (MAUAM). Cuenca, Buenache de la Sierra, R. Gamarra 419 (MAUAM). León, Cármenes, P. Galán 4376 (MAUAM). Teruel, Fortanete, R. Gamarra 439 (MAUAM).

Dactylorhiza foliosa (Rchb. f.) Soó. Portugal: Madeira, Ribeiro Frio, L. Dalgaard & V. Dalgaard 13124 (C).

Dactylorhiza fuchsii (Druce) Soó [=*D. maculata* subsp. *fuchsii* (Druce) Hyl.] Andorra: Coll d'Ordino, R. Gamarra 317 (MAUAM). Denmark: Lolland, Vindeby Skov, K. Wiinstedt (C). Spain: Cuenca, nacimiento del río Cuervo, R. Gamarra 169 (MAUAM).

Dactylorhiza incarnata (L.) Soó. Denmark: Sjaelland, Hedehusene, H.A. Pedersen 778 (C). Russia: Kola Peninsule, I. Blinova 62 (MAUAM). Spain: Ávila, Serranillos, D. Sánchez Mata (MAF 118725). León, puerto de Somiedo, P. Galán 4372 (MAUAM). León, Puebla de Lillo, P. Galán 4378 (MAUAM). León, Meroy, R. Gamarra 623 (MAUAM).

Dactylorhiza insularis (Sommier) Ó. Sánchez & Herrero. France: Corse, Quenza, E.A. Pedersen (C). Spain: Burgos, P. Galán 4474 (MAUAM). Ciudad Real, Los Cortijos de Arriba, R. Gamarra 61 (MAUAM). Madrid, puerto de Canencia, R. Gamarra 280 (MAUAM).

Dactylorhiza maculata subsp. *caramulensis* Verm. Spain: Ávila, Puerto de la Peña Negra, P. Galán 4455 (MAUAM).

Dactylorhiza maculata (L.) Soó subsp. *maculata*. Andorra: Coll de la Botella, R. Gamarra 319 (MAUAM). Denmark: Nordjylland, Laesø, A. Hansen (C). Russia: Kola Peninsule, I. Blinova 7 (MAUAM). Spain: Asturias, Puerto de Leitariegos, P. Galán 4371 (MAUAM). León, Puerto de Tarna, R. Gamarra 334 (MAUAM). León, pinar de Lillo, R. Gamarra 335 (MAUAM). Lérida, Alt Aneu, R. Gamarra 215 (MAUAM).

Dactylorhiza majalis (Rchb.) P.F. Hunt & Summerh. subsp. *majalis*. Denmark: Aeroe, Aeroeskobing, A. Hansen (C). Spain: Lérida, Alto Arán, R. Gamarra 217 (MAUAM). Lérida, Alins, R. Gamarra 320 (MAUAM). Lérida, puerto de la Bonaigua, R. Gamarra 458 (MAUAM).

Dactylorhiza purpurella (T. Stephenson & T.A. Stephenson) Soó. Denmark: Nordjylland, Hunstrup, Dahl (C).

Dactylorhiza romana subsp. *guimaraesii* (E.G. Camus) H.A. Pedersen [= *D. sulphurea* (Link) Franco]. Spain: Cáceres, Castañar de Ibor, P. Galán 4403 (MAUAM). Lugo, Rivas del Sil, R. Gamarra 608 (MAUAM).

Dactylorhiza romana (Sebast.) Soó subsp. *romana*. Greece: Samos, Or. Ampelos, H.A. Pedersen 776 (C).

Dactylorhiza sambucina (L.) Soó. Denmark: Sjaelland, Faarevejle, H.A. Pedersen 777 (C). Greece: Nom. Kastorias, Grammos, Hartvig & al. 7168 (C). Spain: León, Cabrillanes, R. Gamarra 618 (MAUAM). Lérida, Vall de Mulleres, R. Gamarra 331 (MAUAM). Teruel, Albarracín, P. Galán 4190 (MAUAM).

Dactylorhiza viridis (L.) R.M. Bateman, Pridgeon & M.W. Chase [=*Coeloglossum viride* (L.) Hartm.] Canada, Québec, Tewkesbury, F. Marie-Victorin 45237 et al. (US 3614760). Russia: Kola Peninsule, I. Blinova 68a (MAUAM). Spain: Barcelona, Castellar de N'Hug, sine col. (MA 328766). Huesca, Benasque, sine col. (MAF 37102). USA, Maine, Fort Kent, K. Mackenzie 3584 (US 648754). USA, South Dakota, Harvey Peak, W.H. Over 12445 (US 1366192). USA, Vermont, Westmore, E.H. Eames & C.C. Godfrey 9376 (US 3021875).

Gymnadenia austriaca (Teppner & E. Klein) P. Delforge [=*Nigritella nigra* subsp. *austriaca* Teppner & E. Klein]. Germany: Bayern, Landkreis Rosenheim, Grosser Traithen, A. Hanak 03/68 (M).

Gymnadenia austriaca var. *gallica* (E. Breiner & R. Breiner) P. Delforge [=*Nigritella austriaca* subsp. *iberica* (Teppner & E. Klein) L. Sáez. Spain: Huesca, Chía, P. Galán 4463 (MAUAM). Lérida, Vall de Mulleres, R. Gamarra 326 (MAUAM).

Gymnadenia conopsea (L.) R. Br. Russia: Kola Peninsule, I. Blinova 65 (MAUAM). Spain: Asturias, San Miguel, B. Lázaro (MAF 36725). Barcelona, Fígols, R. Gamarra 406 (MAUAM). Burgos, Portillo de Lunada, P. Galán 4097 (MAUAM). Huesca: Chía, P. Galán 4461 (MAUAM). Lérida, Puerto de la Bonaigua, R. Gamarra 453 (MAUAM). Sweden: F.R. Fosberg (US 2683427).

Gymnadenia corneliana (Beauverd) Teppner & E. Klein [=*Nigritella nigra* subsp. *corneliana* Beauverd]. France: Hautes Alpes, Lautaret, P. de Palisieux (M). France: Alpes Maritimes, Col de Tende, E. Dorn 300 (M).

Gymnadenia densiflora (Wahlenb.) A. Dietr. Germany: Westfalen, Hamm (W 1994: 03286).

Gymnadenia miniata (Crantz) Hayek [=*Nigritella rubra* (Wettst.) K. Richt.]: Austria, Tirol, Ailberg, H. Merxmüller (M). Italy, SüdTirol, H. Roessler 629 (M).

Gymnadenia nigra (L.) Rchb. f. [=*N. nigra* (L.) Rchb. f.]: Austria: Molnia, G. Schellenberg (M). France: Hautes Alpes, Aiguilles, D. Podlech 24526 (M). Switzerland, Wallis, Hildebrand (M).

Gymnadenia odoratissima (L.) Rich. Austria: Tuxer Alpen, Sankt Jodok, A. Polatschek (W 1978: 13087). Bad Vöslau, H. Fleischmann (W 1968: 18743).

Pseudorchis albida (L.) Á. Löve & D. Löve. Russia: Kola Peninsule, I. Blinova 90 (MAUAM). Spain: Lérida, Vall de Mulleres, R. Gamarra 329 (MAUAM).